GEORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

[X] ORIGINAL [ ] REVISION NO. _____

Project No./(Center No.) G-33-517 (P5052-0A0) GTRC/~~GTX~~ DATE 7 / 27 / 87

Project Director: Dr. Fred L. Suddath School/~~Lab~~ Chemistry

Sponsor: NASA Headquarters, Washington, DC

Agreement No.: Training Grant No. NGT-50208

Award Period: From 6/1/87 To 5/31/88 (Performance) 5/31/88 Reports

Sponsor Amount:	New With This Change	Total to Date
Contract Value: $		$ 18,000
Funded: $		$ 18,000

Cost Sharing No./(Center No.) _____ Cost Sharing: $ _____

Title: NASA Graduate Student Researchers Program

ADMINISTRATIVE DATA

OCA Contact E. Faith Gleason x4820

1) Sponsor Technical Contact:

Jackie Counts Code: XEU
NASA Headquarters
Office of External Relations
Educational Affairs Division
Washington, DC 20546

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Alfred Wilson Code: HWC
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Contracts & Grants Division
Washington, DC 20546

Military Security Classification: N/A
(or) Company/Industrial Proprietary: N/A

ONR Resident Rep. is ACO: _____ Yes X No
Defense Priority Rating: N/A

RESTRICTIONS

See Attached _____ Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval — Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: ~~Title vests with~~ The use of training grant funds for the purchase of equipment will not be permitted.

COMMENTS:

SPONSOR'S I.D. NO. 02.105.002.87.010

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FORM OCA 65:1086

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 02/18/91

Project No. G-33-517 Center No. P5052-0A0

Project Director SUDDATH F L JR School/Lab CHEMISTRY

Sponsor NASA/HEADQUARTERS/WASHINGTON, DC

Contract/Grant No. NGT-50208 Contract Entity GTRC

Prime Contract No.

Title NASA GRADUATE STUDENT RESEARCHERS PROGRAM

Effective Completion Date 900831 (Performance) 900831 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	Y	
Final Report of Inventions and/or Subcontracts	Y	
Government Property Inventory & Related Certificate	N	
Classified Material Certificate	N	
Release and Assignment	N	
Other	N	

Comments

Subproject Under Main Project No.

Continues Project No.

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
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NOTE: Final Patent Questionnaire sent to PDPI.

G-33-517

Abstract:

The first and often most difficult step in determining the structure of a molecule is to obtain crystals that meet the requirements of X-Ray diffraction. The physics of crystal growth of small molecules is well understood; while some of the knowledge is applicable, the macromolecular crystal growth process is clearly more complex. An instrument has been developed that intelligently controls certain solution parameters allowing the importance of each parameter to be evaluated.

The first element being studied is the rate of approach to critical supersaturation. The device employs N_2 (g) to control the evaporation of water from drops suspended from silanized microscope coverslips. A thermal conductivity detector is used to monitor the amount of water leaving the drops and provides closed-loop control of the evaporation process. Data acquisition and control are accomplished with LabVIEW software (National Instruments) on a Macintosh II microcomputer. Crystals of the enzyme lysozyme have been grown at different evaporation rates and analyzed according to size, morphology and number of single crystals.

Other variables of importance to the optimization of the protein crystal growth process are pH, temperature, and diffusoconvective mixing in solution. Experiments are underway to adapt this device to study these parameters and their importance to protein crystal growth. Development of a Laser Light Scattering (LLS) technique for detection of crystal nucleation is also underway. Such a technique will allow closer investigation of each step in the crystal growth process.

Past Year's Research Accomplishments:

I. Revised Control and Monitoring Network
   A). Hardware
   B). Software

II. Design of Crystallization Cell

III. Drop Monitoring
   A). Relative humidity measurements
   B). Thermal Conductivity Detector (TCD)
   C). Laser Light Scattering (LLS)

IV. Crystal Growth Experiments
   A). Survey of crystallization conditions
   B). Effect of rates of approach to supersaturation

I. Due to serious limitations of the previously described experimental set up [1] it was necessary to update to a more versatile control and monitoring network. Changes were made in both the hardware and software components (FIG 1).

I. A). The Apple Macintosh II was chosen for its significant advantages over the AT&T personal computer. Namely, more sophisticated system software, ability to run multiple application programs simultaneously and a faster central processing unit. The Mac II is equipped with a CMS Enhancements, Inc. 60M hard drive and a color monitor. Two interface boards were purchased from National Instruments (Austin, Texas). The NB-DIO-24 is a 24-bit parallel digital interface for the Macintosh II computer. It is a general purpose peripheral interface containing 24 programmable I/O pins to allow communication with a variety of equipment. Its primary use is to output a signal to the PB16A (opto 22) I/O mounting rack to operate relays for the control of the micro-solenoid

valves (FIG 1). Also in use is a multifunctional analog, digital and timing I/O board (NB-MIO-16) with a 12-bit A/D converter with 16 analog inputs, two 12-bit D/A converters with voltage outputs, eight digital I/O pins and three 16-bit timer channels. The NB-MIO-16 is used to monitor signals from both the humidity indicators (Thunder Scientific) or the Gowmac thermal conductivity detector providing closed loop control of the drop evaporation process. This data acquisition and control system is very versatile and will not be a limiting factor for future research directions.

I.B). Also purchased from National Instruments was the graphical programming language LabView which provides tools for constructing custom user interfaces for data acquisition and control. LabView was used to develop the current software system (FIG 2).

II. It was necessary to redesign the crystallization cell to meet the requirements of proper gas flow, control and ease in drop formation. The current cell is shown in figure 3. The cell has twelve crystallization wells each employing a separate micro-solenoid valve (Lee Scientific) to control gas flow. Two major advances were made with the new design. First, the drops are placed on silanized glass microscope coverslips. The coverslips are then inverted and sealed to the well with grease as is done in the traditional hanging drop procedure. Second, each individual well is made of glass thereby minimizing moisture absorption and gas permeation which was a problem with earlier designs. Test were carried out to assure each well was air tight. The flow through each well was found to be equal within 2%. Furthermore, the gas pressure required to break the seal between the coverslips and the glass well was greater than 3 psi which is well above the required working pressures.

III. In order for a system to meet the I.U.P.A.C. definition of automated at least one major operation must be controlled without human intervention by a feedback system [2]. Three on-line detection mechanisms have been examined. Two provide monitoring of drop

solute concentrations and a third (LLS) to provide detection of crystal nucleation.

III.A). Relative humidity measurements within the crystallization well are carried out with a humidity indicator that provides voltage readings proportional to relative humidity. A typical response from the indicator is shown in figure 4 for a reasonable gas on/off sequence. The advantages of using this indicator are its small size which allows it to be incorporated easily into the cell and it is relatively inexpensive. It does suffer, however, from a slow response time and low sensitivity relative to the thermal conductivity detector.

III.B). The thermal conductivity detector provides a sensitive measure of the moisture leaving the cell with the nitrogen carrier gas. These types of detectors have been used extensively in the past to quantitate amounts of various compounds [3]. The response of the TCD to different gas purges is shown in figure 5. While the TCD allows determination of water leaving a single cell it will be difficult and expensive to multiplex it to monitor several cells simultaneously.

III.C). Recently, a Model 127 Stabilited He/Ne Laser was purchased from Spectra-Physics along with a Model 875 Photodiode detector (Newport). Work is just beginning on the design of an optics set-up that allows detection of changes in the intensity of scattered light as nucleation occurs. The thought is that once nucleation is detected changes made in the supersaturation conditions may be desired to slow post-nucleation growth allowing crystals to grow slowly with less defects. Considerable thought is being put into the design of an optical cell that allows deployment of a drop, air tight seal, a water jacket for temperature control and gas inlet and outlet ports.

IV. Preliminary results indicate that the rate of approach to supersaturation is an important factor in determining relative number and size of crystals. The protein of choice was the enzyme

lysozyme whose crystal growth processes have been studied by several researchers [4-7].

IV.A). A survey of various crystal growth conditions was carried out to determine the initial conditions of pH, lysozyme concentration, salt concentration and buffer concentrations. Lysozyme was obtained from Sigma (3X crystallized, dialyzed and lyphilized). To remove salts 5% (w/w) solutions of lysozyme were dialyzed against deionized water overnight and lyphilized. The lysozyme concentration was determined spectrophotometrically from the absorbance at 280 nm, using the value $A^{1\%}(1cm) = 26.35$ [8]. Conditions were scanned using the vapor diffusion method of crystal growth in a Linbro box [9]. Crystals were grown at 22°C in an insulated box to minimized temperature fluctuations. Conditions yeilding best crystals were 50 mg/ml Lysozyme and 5% (w/w) NaCl both made in 0.1M Sodium Acetate Buffer at pH 4.00 (+/- 0.02). These are the initial conditions used for all experiments below.

IV.B). Crystals have been grown in the experimental device at different equilibration rates. The evaporation of water from the drop was controlled with gas purges. Purges were more frequent for fast equilibration rates. Experiments are still being carried out on a complete set of equilibration rates. The most striking trend seen to date is the relationship between the number of crystals per well and the average area (FIG 6). The greater the number of crystals per well the smaller their size. In fact, the largest crystals are the ones with only 1 crystal per well. This is in agreement with Kam, Shore and Feher [10] who did studies on the cessation of growth of lysozyme crystals. Crystals grown at different approach rates to supersaturation differ in size and number (FIG 7). More quantitative work needs to be done to confirm these results.

Objectives for the Next Research Period:

I. Complete crystal growth experiments described in IV.B).

II. Develop LLS procedure

III. Evaluate the effect of changes in conditions after nucleation

IV. Investigate effect of other parameters on crystal growth
A). pH
B). Temperature

Objectives I., II., III. have been discussed above.

IV. Proteins are known to vary in solubility with changes in pH and temperature. These two variables have been used as tools for crystallization in the past [9]. Previous results have shown the pH of the drop can be changed with purges of acidic or basic saturated nitrogen gas [1]. Experiments will be carried out using pH to bring the solution to a point of supersaturation and the resulting crystals will be evaluated according to size, number and quality. The use of temperature changes as a means for lowering the protein solubility will also be examined.

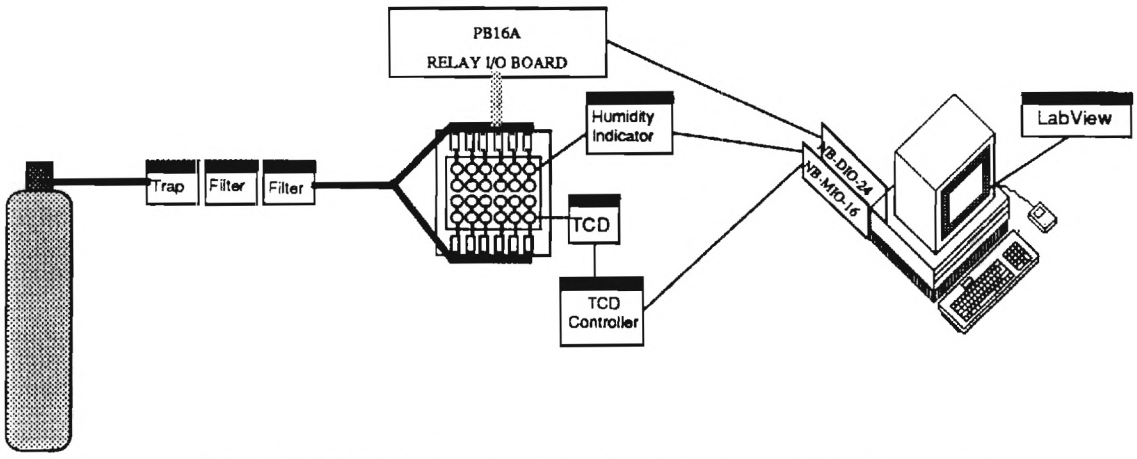


Figure 1. Schematic of control and monitoring network. In-line trap captures moisture. First filter is 60 micron and second is 0.5 micron. See text for details.

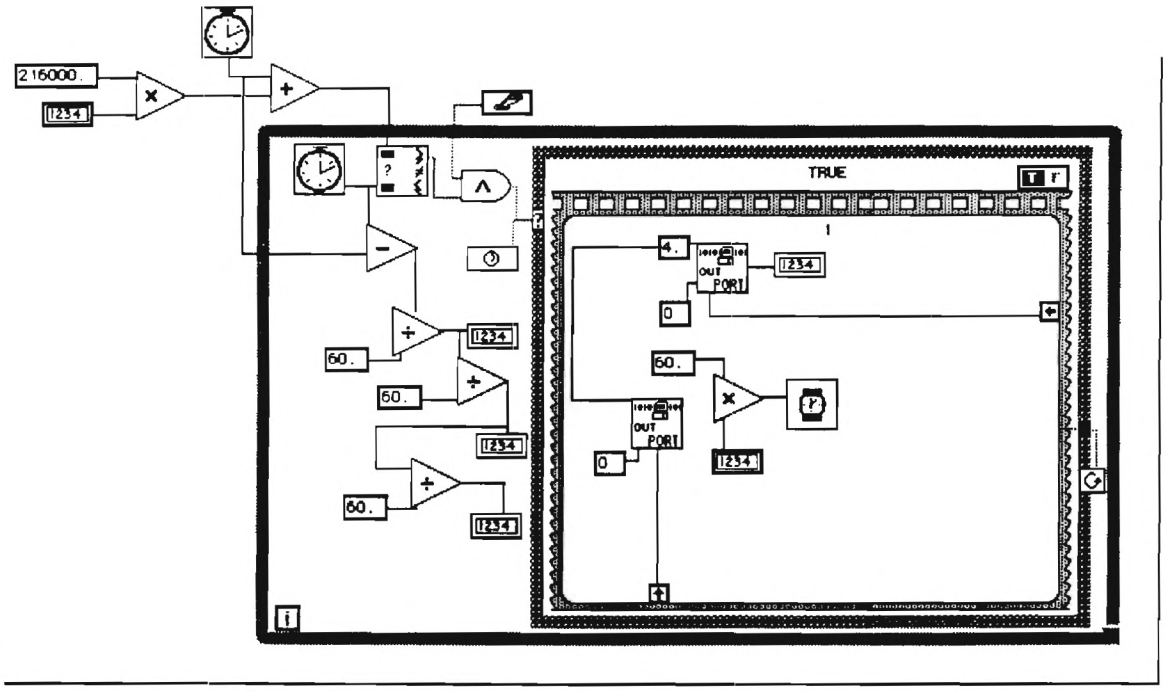


Figure 2. An example of the graphical program language Labview. This program oversees the digital output signals to the PB16A board. There is also a timing sequence as well as a panic button to terminate program and shut off valves.

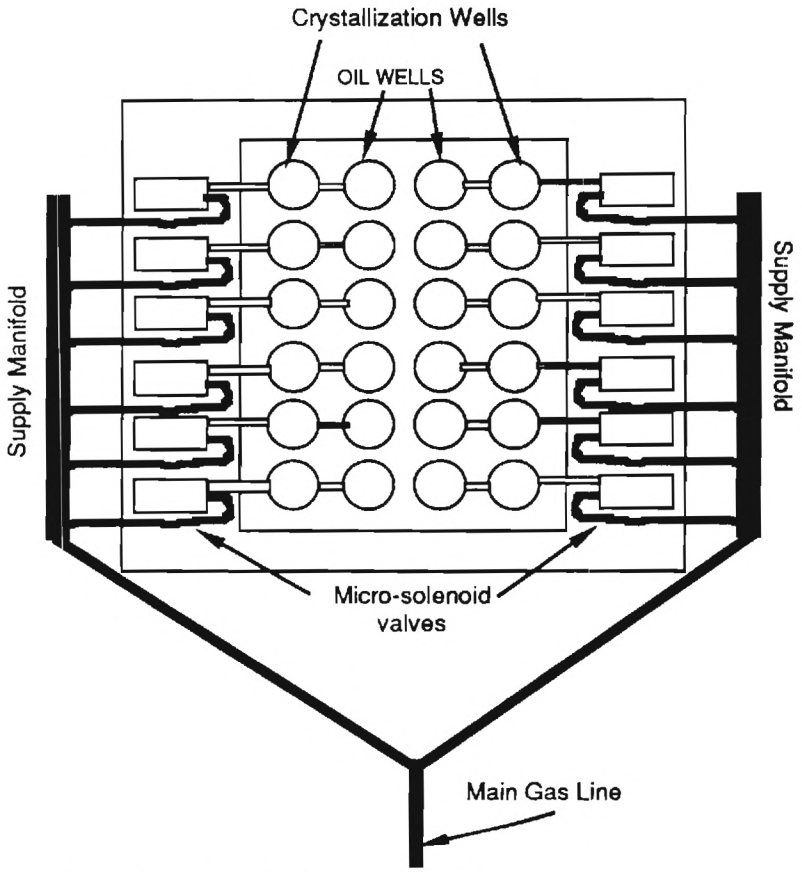


Figure 3. Schematic of crystallization cell.

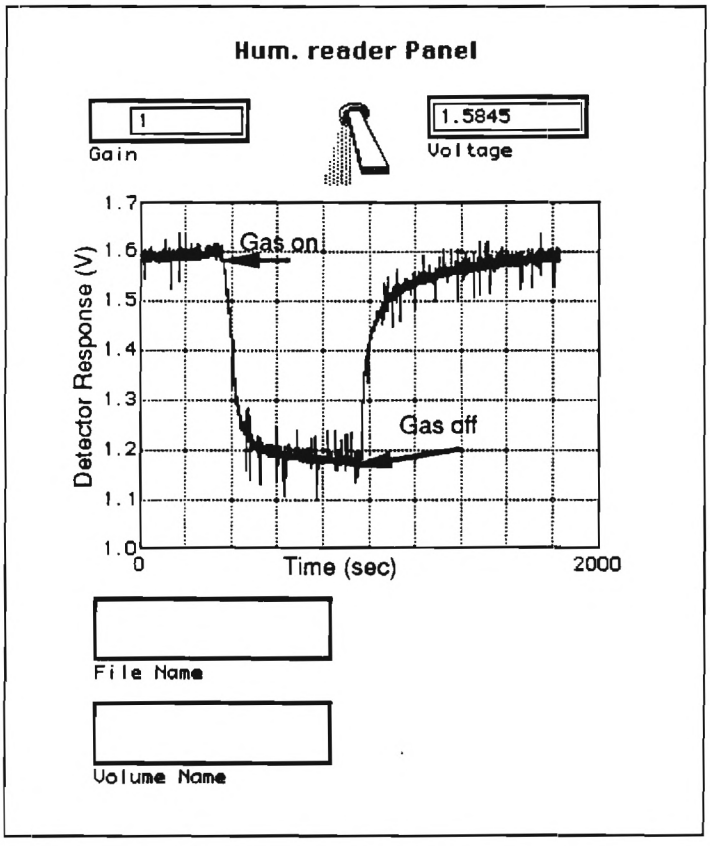


Figure 4. An example of the humidity indicator readout. An increase in voltage represents and increase in relative humidity within the cell.

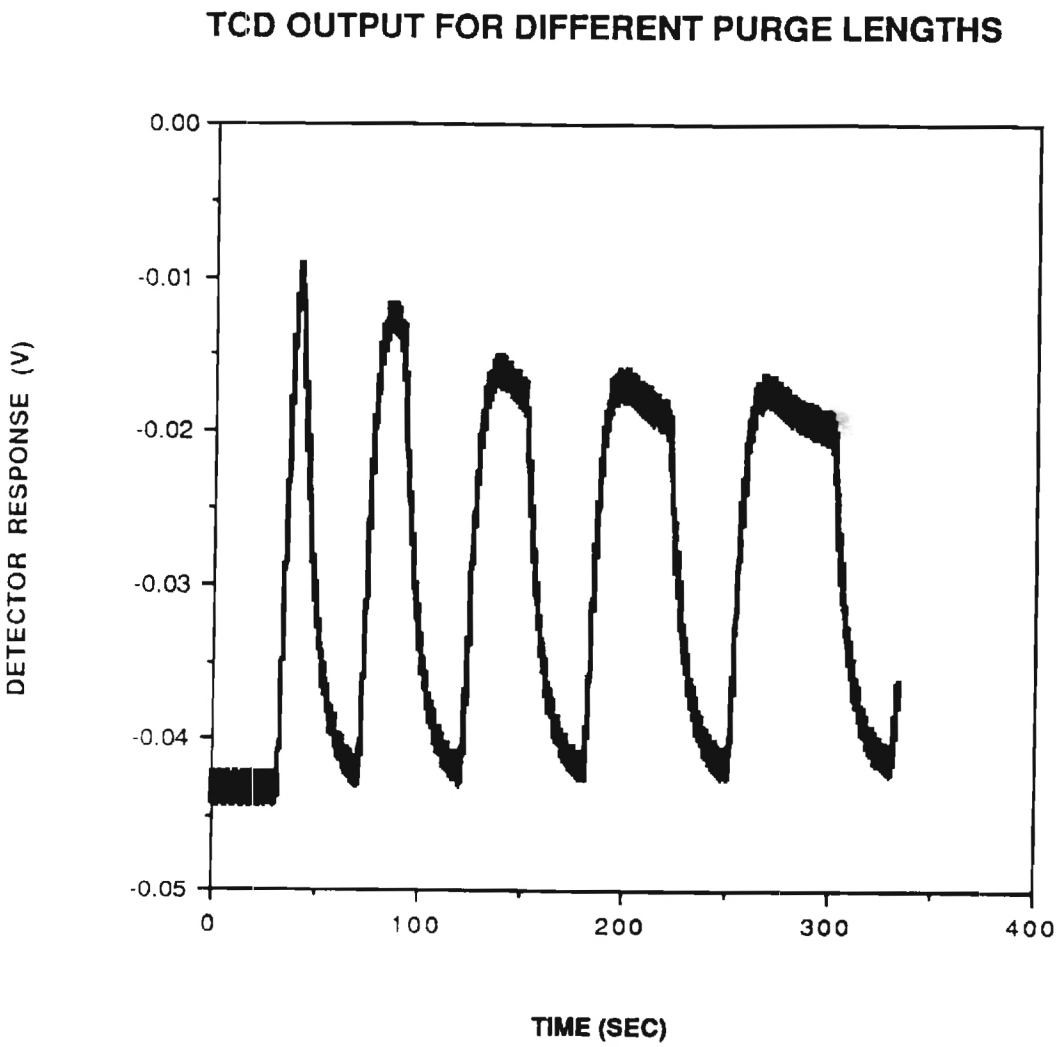


Figure 5. Typical response of thermal conductivity detector.

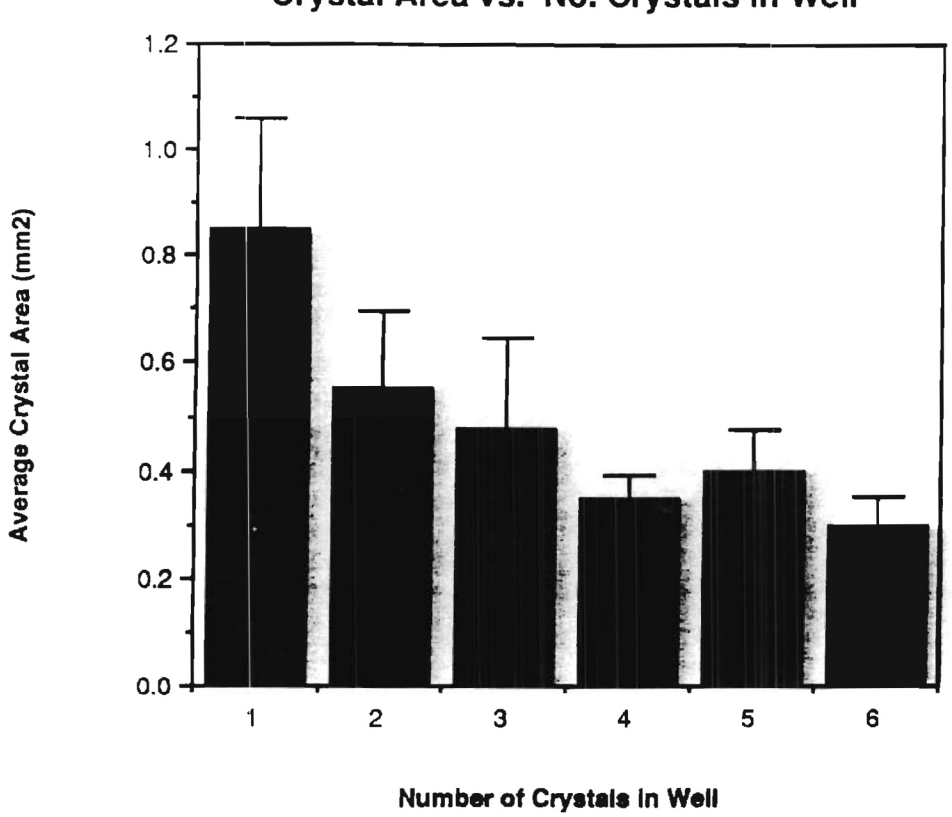
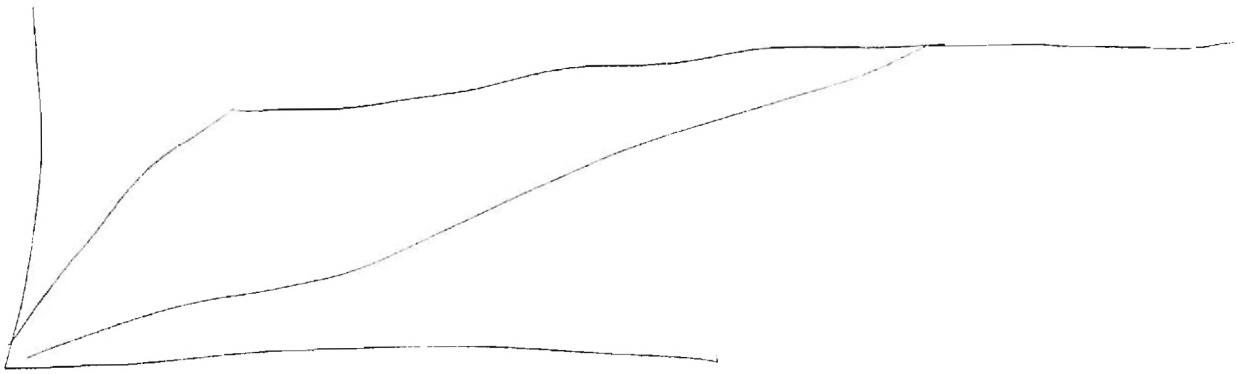


Figure 6. Average crystal area decreases with number.

a). curve 1

b). curve 2

Figure 7. Size and number of crystals shown to vary with type of evaporation curves.



References:

1. Wilson, L.J. and F.L. Suddath, NASA Graduate Student Research Proposal Renewal, 1988.

2. Skoog, D.A., (1985) *Principles of Instrumental Analysis*, 3rd ed, Saunders College Publishing, Philadelphia.

3. Richmond, Adah B., J. Chrom. Sci., **9**, 92-98 (1971).

4. Durbin, S.P. and G. Feher, J. Crystal Growth, **76**, 583-592 (1986).

5. Ataka, Mitsuo and Tanka, Biopolymers, **25**, 337-350 (1986).

6. Pusey, Marc and Robert Naumann, J. Crystal Growth, **76**, 593-599 (1986).

7. Ries-Kautt, M.M., and F. Ducruix, J. Biol. Chem., **264**, 2, 745-748 (1989)

8. Sophianopoulos, A.J., C.K. Rhodes, D.N. Holcomb and K.E. Van Holde, J. Biol Chem., **237**, 1107 (1962).

9. McPherson, A. (1982) *Preparation and Analysis of Protein Crystals*, John Wiley and Sons.

10. Kam, Z., H.B. Shore and G. Feher, J. Mol. Biol., **123**, 539-555 (1978)

Schedule:

We expect the proposed research plan to begin June 1, 1989. The proposed research/study plan will conclude in approximately 1 year. During this period Ms. Wilson will be expected to complete her requirements of the Analytical Division. In addition she will pursue a plan of research outlined in this renewal. We expect that visits to the MSFC in Huntsville, Alabama will be both desirable and necessary. These visits will be scheduled at times that do not interfere with class meetings at Ga. Tech and fit the schedules of Drs. Snyder and Carter at MSFC when possible. Dr. Suddath is in weekly contact with Drs. Snyder and Carter on other matters of mutual interest thus communications with MSFC is routine. We expect both visits to MSFC and telephone conversations to be essential for the success of this project.

Dr. Suddath and Ms. Wilson will make the necessary arrangements to attend the requisite meetings in Washington, D.C.

Budget:

Student stipend		$12,000
Tuition and Fees	$ 1500	
Travel / Lodging to MSFC	$ 1500	
Subtotal		$ 3,000
University Allowance		
Travel / Lodging to Wash. D.C.		$ 3,000
		$18,000

APPROVAL:

Faculty Advisor: Dr. F.L. Suddath

_____

Director of School of Chemistry
Dr. Robert Pierotti

_____

School of Chemistry
(404) 894-4002

Georgia Institute of Technology
Atlanta, Georgia 30332
A Unit of the University System of Georgia

January 26, 1989

Mr. Joseph K. Alexander
Assistant Associate Administrator
Office of Space Science and Applications
Code E
National Aeronautics and Space Administration
Washington, DC 20546

Dear Mr. Alexander:

This letter is written in support of the application by Miss **LORI WILSON** for renewal of a NASA fellowship. Miss Wilson has completed the coursework requirements for a Ph.D. in chemistry achieving a GPA of 3.7. She has successfully passed the written comprehensive examination in her area of specialization. In addition, she gave an excellent presentation of her research in the seminar required of all graduate students in our program during her second year in residence.

The following requirements are left: oral defense of an original research proposal, completion of her dissertation and the successful defense of this work. Miss Wilson's progress in fulfilling the doctoral degree requirements is on track with previous students who earned their Ph.D.'s in four years.

If further information is required, please contact me at (404) 894-4014.

Sincerely,

Lawrence A. Bottomley
Associate Professor of Chemistry

PAST YEAR'S ACCOMPLISHMENTS:

I. Crystallization cell design.

A). Droplet Dispenser
B). Computer interface

II. pH control and monitoring.

III. Determination of pH equilibration within a Linbro cell.

IV. Development of equilibration control theory.

I. It was necessary to design a crystallization chamber that met the requirements for proper gas flow, control, compact size, and ability to incorporate new modifications. The design we are currently using incorporates printed circuit technology to eliminate the need for gas inlet and outlet tubing minimizing leakage problems. The cell has six crystallization compartments each employing a micro-solenoid valve to control gas flow (Fig. 1). This building of prototype hardware, testing and redesign was quite time consuming; however, it was necessary to arrive at the current cell. The current cell allows us to evaluate a number of crystallization parameters in an accurate and reproducible manner.

I. A). Considerable research and thought was necessary to design a droplet dispenser that provided uniform drop formation, volume measurement and met the air tight requirements. We employ dispensers of two different types. The first is a combination of a teflon dispenser tip and an accurate microliter syringe. This allows for calibration of the crystallization cell and determination of evaporation rate of the drop. The second dispenser is used exclusively with crystal growth experiments because it allows the drop to be stored in the dispenser tip before opening the crystallization compartment.

I. B). The crystallization cell was designed to be controlled by a PC compatible AT&T 6300 interfaced to a Keithly controller and run by Labtech Notebook software. The solenoid valves are controlled by a waveform file generated on a VAX 11/780 and downloaded to the PC. Work was conducted throughout the past year on refining this control network (Fig. 2)

II. Significant advances have been made in our ability to control the pH of the drop via the gas phase. The ability to flush specific chambers with various gases makes it feasible to control the pH of the crystallization droplet. The droplet dispenser was replace with a micro pH electrode and the pH was monitored by the computer as a function of various acid/base saturated nitrogen gas. The pH was lowered by flushing the chamber with nitrogen in equilibrium with acetic acid solutions and raised with nitrogen in equilibrium with ammonium hydroxide (Fig. 3).

III. There was some concern that the conditions found in the traditional Linbro box could not be mimicked by nitrogen equilibration of the drop. One area that needed to be studied was the transfer of ions between the reservoir in the Linbro box and the hanging drop. Experiments were conducted that determined that often there is transfer of ions between the two solutions. The experimental setup employed of a large pH gradient between the crystallization droplet and the reservoir salt solution. Over time, the pH of the two solutions within the chamber equilibrated indicating movement of ions (Fig. 4). This result requires consideration in our development of a device to obtain protein crystals.

IV. An empirical approach has been developed to investigate the effect of a number of evaporation rates on the size, number and quality of crystals. Evaporation profiles can be generated by software we have developed and translated into valve opening/closing sequences to produce evaporation rate profiles for any multisegment curve (Fig. 5). The constraints on the profiles are bounded only by the minimum evaporation rate when the chamber is sealed. It is possible to have liquid transported from the gas phase to the liquid phase by flushing the chamber with water saturated nitrogen.

Research Plans: The objectives of the next research period are:

1. Continue a systematic survey of the effects of equilibration rate on crystal size and crystal number.

II. Development of procedures to evaporate the water from protein solutions without altering other parameters of the solution such as pH.

III. Development of procedures to use programmed changes in pH as a method of achieving protein saturation.

IV. Develop new procedures to monitor the conditions within crystallization solutions in order to provide a feedback signal to the control computer.

Now that an experimental apparatus has been developed data must be accumulated to support the preliminary indication that evaporation rate is an important factor in determining relative number and size of crystals. Lysozyme will be the standard protein for these experiments since a partial phase diagram of this protein is available for changes in pH, ionic strength, and temperature. There will be at least two approaches to trying to understand the variation in crystals with evaporation rates.

First, the approach mention in IV. above.

Second, a closed loop feedback system where an observation or measurement from the crystallization drop will be interpreted as a signal to alter the current evaporation profile. For example, if a method can be developed (see below) to detect the onset of crystal nucleation then the supersaturation could be changed in a number of ways. The supersaturation could be decreased by, changing the temperature, introducing water saturated nitrogen into the chamber to dilute the protein, or altering the pH. It might be more appropriate to seal the chamber, stopping all evaporation, and let the protein concentration decrease slowly as it leaves the solution phase to the crystals. It is expected that this apparatus will be sufficiently reproducible that once a prenucleation event is detected in one experiment that future or parallel experiments can be programed to follow the same profile.

II. Preliminary experiments have indicated that the evaporation of a crystallization droplet by pure dry nitrogen can result in changes in pH due to the removal of volatile acids or bases from the gas volume that surrounds the crystallization droplet. When the volatile components are removed and the chamber is resealed the equilibrium (in addition to water) between the volatile components in the gas phase and liquid phase must be reestablished. This results in a decrease in the concentration of acid or base in solution resulting in a pH change. We plan to investigate the magnitude of this problem by monitoring ΔpH as a function of buffer type and concentration. If this appears to be a

serious problem we will introduce compensations into the normal evaporation profiles that also control the pH by the appropriate introduction of acid or base.

We are also investigating better methods to monitor the pH of crystallization drops. This might involve the introduction of indicator dyes that will be monitored for spectral changes as a function of pH. This method is generally considered to be very undesirable from the crystallization standpoint; however, in could prove to be a useful method to detect the pH changes in a single monitor droplet that is being controlled in parallel with a number of identical droplets.

III. The use of pH changes to induce supersaturation is rarely used in protein crystallizations. Most proteins, however, have a definite solubility change with changes in pH of the crystallization solution. One of the difficulties of exploiting the parameter has been the complication of reproducibly changing the pH over a time period. The apparatus we have designed is capable of changing the pH of the crystallization droplet automatically by following a preprogramed pH profile. We plan to investigate the usefulness of varying this parameter with a number of test proteins provided by Dr. Alexander McPherson and with lysozyme. Dr. Edward Meehan has determined the phase diagram of lysozyme as a function of pH at a number of temperatures and ionic strengths. By saturating a solution with lysozyme at a particular pH then changing the pH toward supersaturation we can investigate the types, sizes and quality of crystals using these new crystallization procedures.

It is our intention to ascertain the suitability of using changes in pH as a general crystallization strategy. Frequently preliminary surveys of crystallization conditions vary the pH in an empirical stepwise fashion to find the proper combination of complementary surface charges and protein solubility. In many cases one ultimately finds that the quality and size of the crystals are very sensitive to small changes in pH. This fact suggest that a survey of the effects on crystallization of changes in pH might be more quickly determined with a smoothly varying carefully controlled pH change.

IV. A survey of biophysical methods that are applicable to detecting crystal nucleation events indicate that laser light scattering (LLS) is the most suitable method. It does have a number of complications not the least of which is the sensitivity to dust. If one is satisfied to use LLS as a simple indicator that a change in average particle size is taking place it might be usable to indicate nucleation. We will purchase a multi angle LLS

instrument from Wyatt Technology Inc. This LLS unit is relatively inexpensive ($15,000) and most importantly has at least two standard sample cells, a cylindrical cell and a flow cell. We plan to use the flow cell to monitor the state of aggregation in the crystallization solution and use the relative changes in particle size rather than absolute sizes as the signal to monitor. The information about relative particle size will be sent to the control computer for a decision as to whether the supersaturation of the crystallization solution should be altered. Thus providing a form of closed loop control over the evaporation or pH changes that are being used to approach crystallization conditions.

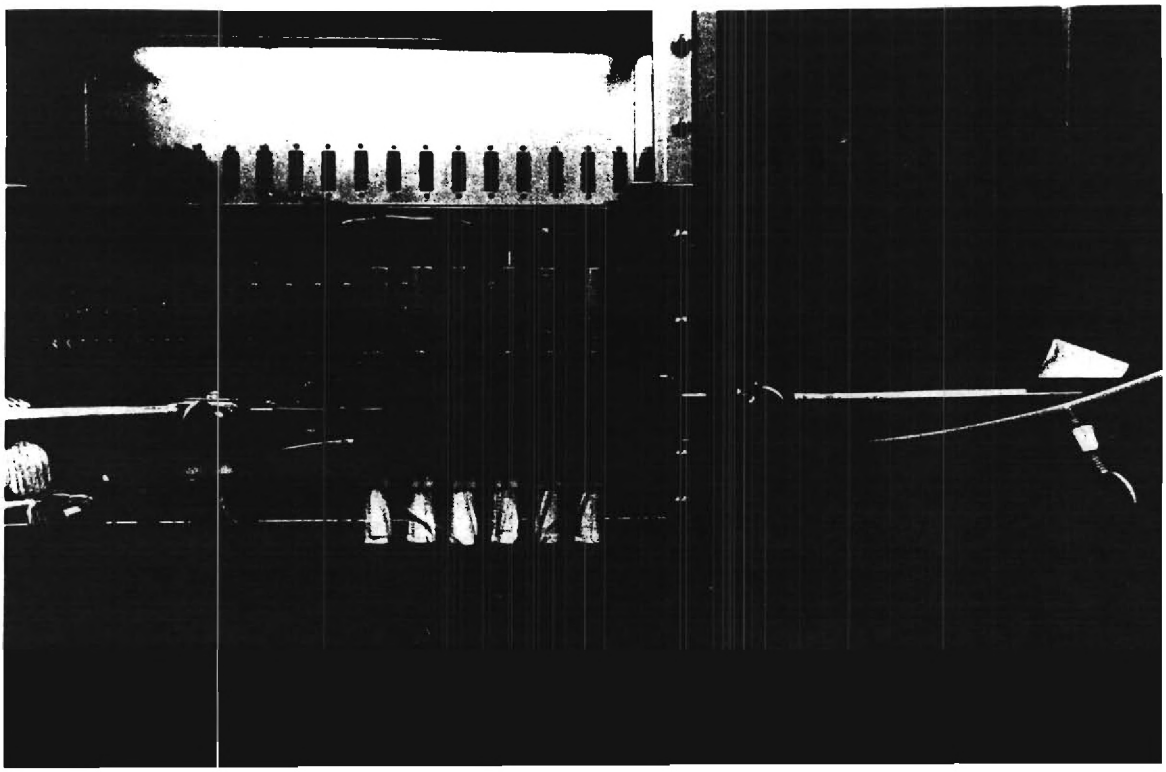


Figure 1. Crystallization Cell and Relay Board.

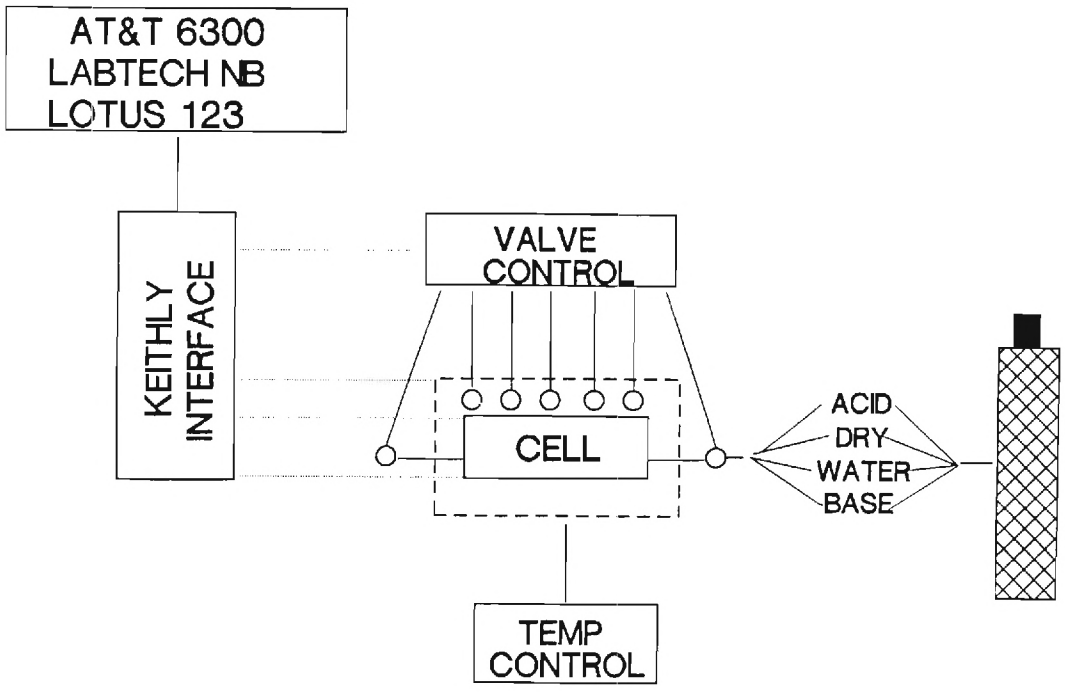


Figure 2. Schematic of control network.

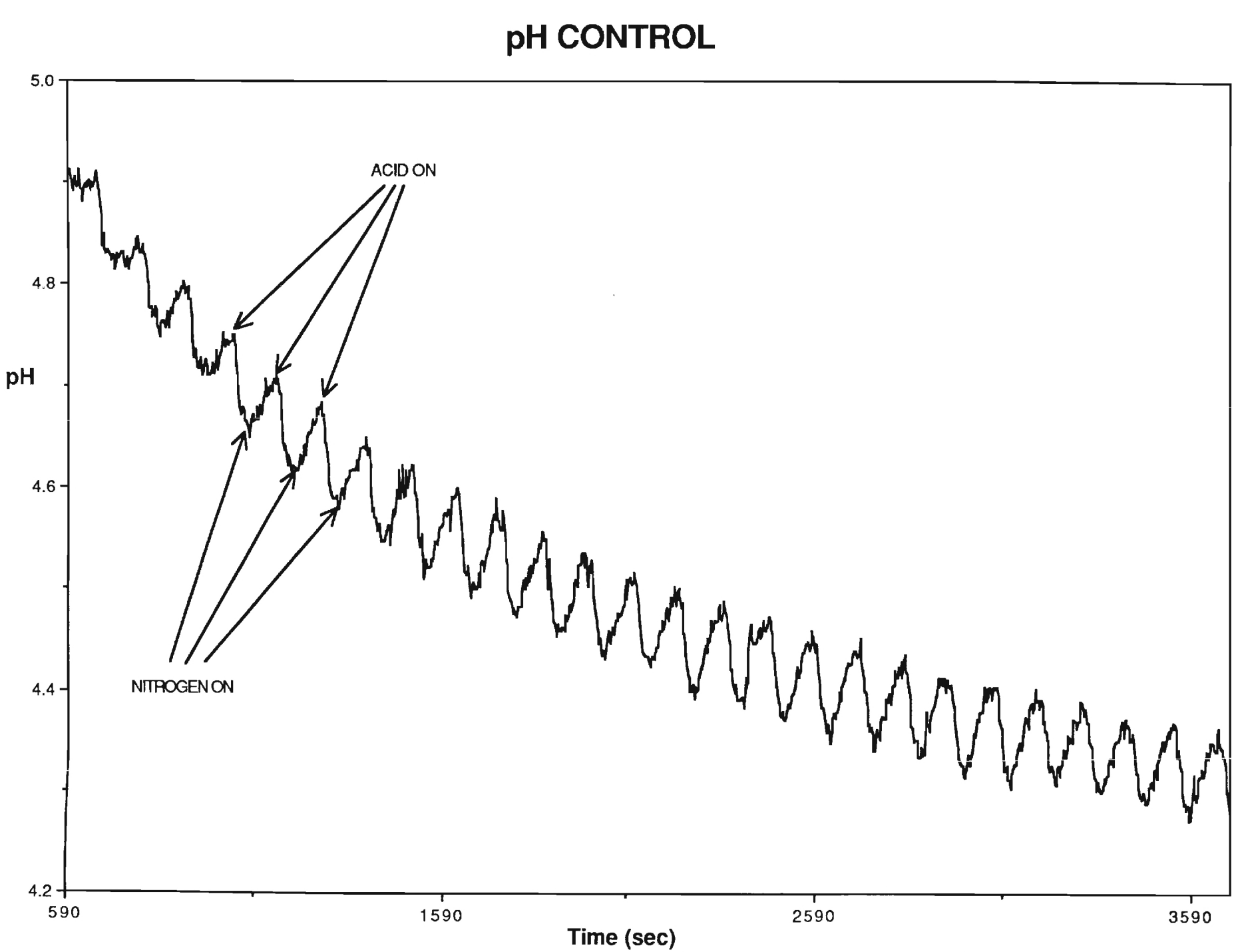


Figure 3. Control of pH of an unbuffered droplet using acetic acid saturated nitrogen gas.

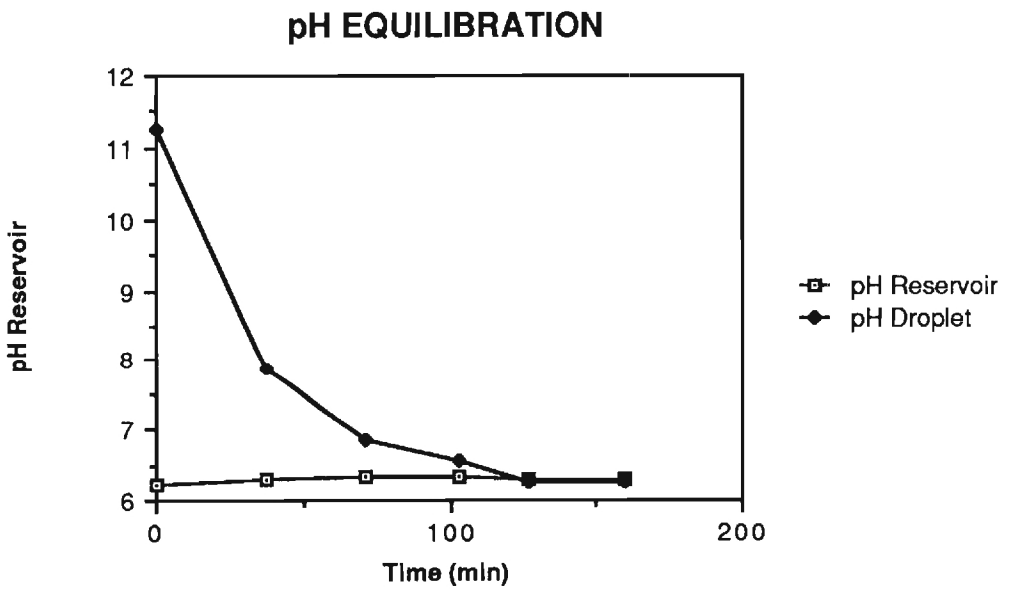


Figure 4. pH equilibration within a Linbro cell.

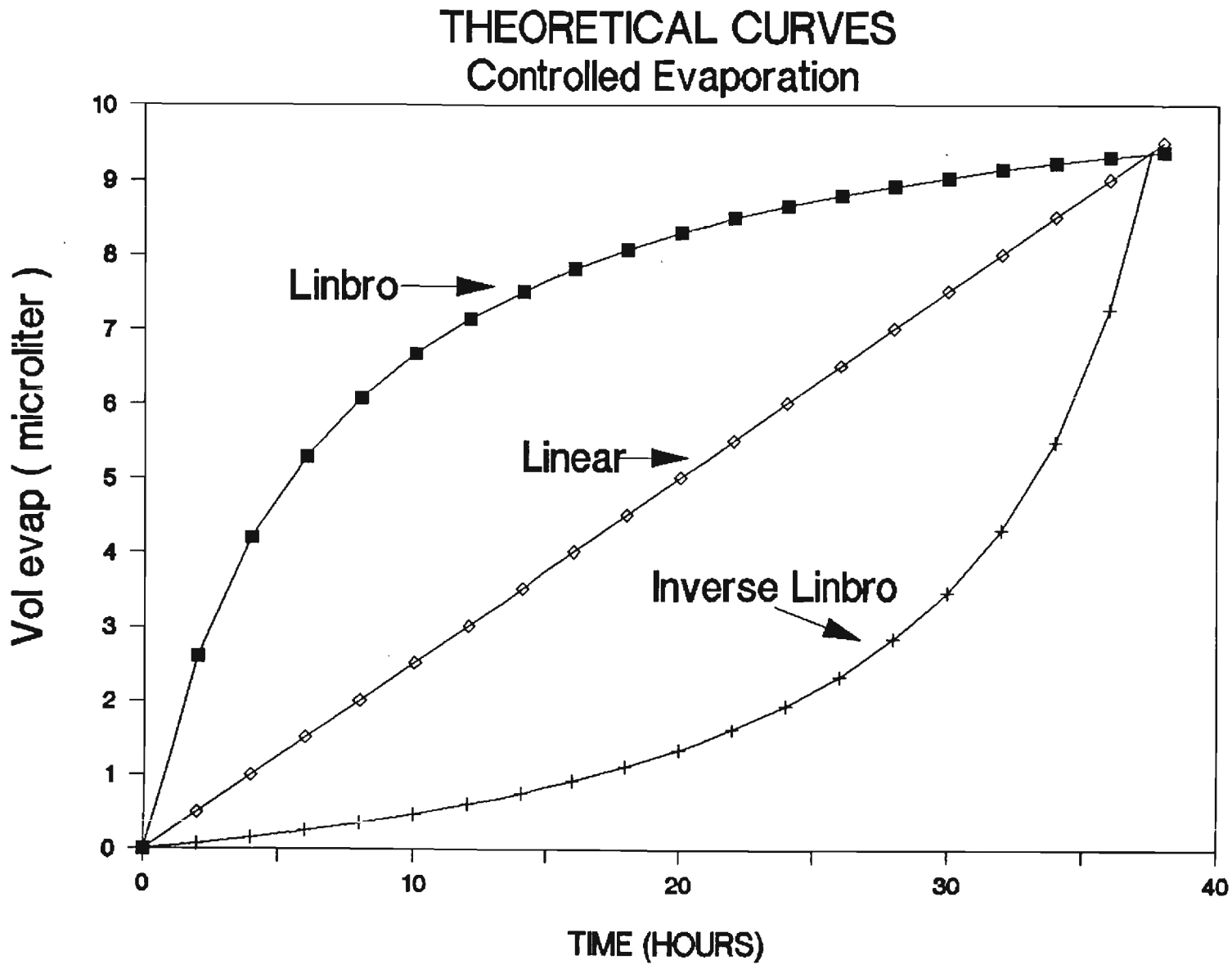


Figure 5. Evaporation curves of a droplet to be investigated.